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UREA HYDROLYSIS IN SOIL

BY

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Urea Hydrolysis in Soil" submitted by William Douglas Gould in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A method for the extraction of urea from soil for analysis has been improved. Various parameters affecting the hydrolysis rate of urea in soil were studied. Under the conditions studied the hydrolysis rate was found to be linearly dependent on concentration. The Arrhenius plot for soil urease was linear from 2°C to 45°C with an experimental activation energy of 9.8 kcal/mole. The urease activity of soil was stimulated by a moderate application of urea but depressed by a heavy application. The reduction in activity can be attributed to the low pH resulting from nitrification of the added nitrogen.

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I. INTRODUCTION

Urea is a high analysis source of fertilizer nitrogen (46%N) and only anhydrous ammonia has a higher analysis (82%N). The cost of urea nitrogen is presently slightly higher than anhydrous ammonia and ammonium sulphate nitrogen. Urea is easy to apply and does not require the expensive application equipment that is necessary for anhydrous ammonia. Recently urea has been used to replace ammonium nitrate in many nitrogen phosphate blends, thereby eliminating certain storage problems that are the result of regulations governing the storage of nitrate fertilizers. For these reasons urea has recently become a popular source of fertilizer nitrogen.

When urea is added to the soil it hydrolyzes very rapidly to ammonia and carbon dioxide. This reaction is catalyzed by the enzyme urease which is produced by many plants and soil microorganisms. The hydrolysis is carried out both by ureolytic microorganisms and free urease in the soil. As the reaction proceeds the pH increases due to the formation of ammonium carbonate from the ammonia and carbon dioxide.

At high rates of urea application, particularly if it is applied with the seed, there can be poor crop responses. Two factors are primarily responsible for some of the problems encountered when urea is used. The first is the loss of nitrogen by volatilization as ammonia since the partial pressure of ammonia in equilibrium with an aqueous solution of ammonium ions increases as the pH increases. The second is the toxicity caused by the ammonia produced at high pH values. The difficulties associated with urea could be reduced if the hydrolysis rate were retarded by a urease inhibitor.

The objectives of this research were: to determine the hydrolysis rate of urea in several Alberta soils; study the effects of temperature, moisture, substrate concentration, and urease inhibitors on the hydrolysis rate in soil; and to assess the changes in urease activity resulting from the addition of urea to the soil.

II. LITERATURE REVIEW

Early Observations

Urea was first isolated by Prout in 1820, and Wohler in 1828 carried out the first synthetic preparation of urea by decomposing ammonium cyanate (Hardesty, 1955). Prior to the industrial synthesis of urea in 1920 natural sources were responsible for all the urea entering the soil. Urea is present in the urine of many mammals and is produced during the decomposition of protein by microorganisms.

The decomposition of urea in the soil was observed during the ammonification of calcium cyanamide (Cowie, 1920) since urea is an intermediate product in this reaction. Using a large number of soils Gibson (1930) measured the rate of urea hydrolysis in the soil and in sterile soil extract solutions inoculated with soil. He found urease activity in every soil tested and the rate of urea hydrolysis to be very rapid compared to other microbial processes. Urease activity was greater in soils of high organic matter content particularly in the surface layers of forest soils.

Properties of Urease

The enzyme urease which catalyzes the hydrolysis of urea was the first enzyme to be isolated. It was crystallized in a relatively pure form from jack bean meal by Sumner (1926).

Sumner and Hand (1928) discovered that minute amounts of the heavy metals will inactivate dilute solutions of urease, but the enzyme can be protected from the denaturing effects of heavy metals by the addition of various colloidal materials.

Urease has a molecular weight of 483,000 (Sumner, Gralen, and Eriksson-Quensel, 1938) and has structural subunits with molecular weights of 83,300 (Reithel, Robbins, and Gorin, 1964). More recently Siegel and Monty (1965) determined the Stokes radius of urease by gel filtration and calculated a molecular weight of 487,000.

Urease prepared by the method of Sumner (1926) or by any of the modified techniques always shows at least two sedimentation peaks in the ultracentrifuge. It has been suggested that the additional peaks are polymers of urease caused by the crosslinking of sulfhydryl groups to form disulphide bonds. Urease solutions high in the monomer of urease (Sumner et al, 1938) were found to have the greatest activities. Creeth and Nichol (1960) were able to obtain one sedimentation peak by reducing the disulphide bonds with sulphite ions. By dialyzing the sulphite treated urease with phosphate buffer they again obtained a sedimentation curve with several peaks which was the same as before the sulphite treatment. Thus they were able to show that the heavier components are polymers of urease and that the polymerization reaction is reversible. Gorin et al, (1962) determined the number of sulphur-containing amino acids in urease and found; 77 methionyl, 29 cystinyl, and 47 cysteinyl residues per urease molecule.

The urease molecule has three types of sulfhydryl groups of differing reactivity (Hellerman, Chinard, and Dietz, 1943). Urease has 20-22 highly reactive sulfhydryl groups per molecule which are not necessary for enzymic activity, another 20-22 that are somewhat less

reactive but essential for activity and 60 relatively inactive groups that are not involved in enzyme activity.

Urease was considered to be specific for urea, but recently has been shown to be capable of hydrolyzing hydroxyurea (Fishbein, Winter, and Davidson, 1965). This reaction differs from the hydrolysis of urea in that only a small amount of hydroxyurea is hydrolyzed because the enzyme is rapidly inactivated. Hydroxyurea is both a substrate and irreversible inhibitor for urease, and one of the reaction products, hydroxylamine, is a reversible inhibitor of urease (Fishbein and Carbone, 1965).

Mechanism and Kinetics of Urea Hydrolysis

Most enzyme catalyzed reactions are bimolecular, but hydrolysis can be treated as a single substrate reaction because the other reactant, water, is generally present in excess so its concentration remains relatively constant during the reaction (Ashmore, 1963). A scheme in which the enzyme (E) and substrate (S) form an intermediate complex can be formulated:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

The constant k_1 is the rate constant for the formation of the ES complex and the constant k_2 is the rate constant for the decomposition of the complex back to enzyme and substrate. The rate constant for the irreversible decomposition of the ES complex to enzyme and products is k_3.

For many cases the enzyme concentration can be assumed to be much lower than the substrate concentration, and the concentration of

enzyme-substrate complex rapidly reaches a stationary value. This is called the steady-state approximation.

$$\frac{d[ES]}{dt} = k_1[E][S] - k_2[ES] - k_3[ES] = 0$$

Initial rates are usually used because most enzymes are inhibited by the products of the reactions they catalyze (Dixon and Webb, 1958). The initial substrate $[S]_o$ and enzyme $[E]_o$ concentrations are related to the steady-state concentrations in the following manner:

$$[E] = [E]_o - [ES]$$

$$[S] = [S]_o - [ES]$$

Since $[E]_o$ is much smaller than $[S]_o$ and $[ES]$ is less than $[E]_o$ then:

$$[S]_o - [ES] = [S]$$

$$\frac{d\,[ES]}{dt} = k_1[S]_o([E]_o - [ES]) - k_2[ES] - k_3[ES] = 0$$

$$[ES] = \frac{k_1[S]_o[E]_o}{k_2 + k_3 + k_1[S]_o}$$

The reaction rate is equal to the rate of formation of products [P] which is also equal to the rate of decomposition of the intermediate complex $k_3[ES]$.

$$V_o = \text{initial reaction rate} = \left[\frac{d[P]}{dt}\right]_o = k_3[ES]$$

Thus the initial rate can be expressed in terms of the initial concentrations of enzyme and substrate.

$$V_o = \frac{k_1k_3[S]_o[E]_o}{k_2 + k_3 + k_1[S]_o} = \frac{k_3[E]_o}{1 + \frac{k_2 + k_3}{k_1[S]_o}}$$

At high initial substrate concentrations the rate tends toward a maximum value $V_{max} = k_3[E]_o$.

$$V_o = \frac{V_{max}}{1 + \frac{K}{[S]_o}} \qquad K = \frac{k_2 + k_3}{k_1}$$

$$\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{K}{V_{max}\,[S]_o}$$

A plot of $1/V_o$ vs $1/[S]_o$ will give a straight line of slope K/V_{max} and an intercept of $1/V_{max}$ on the $1/V_o$ axis. This is called a Lineweaver-Burke plot.

The first rate equation to be published was formulated by Michaelis and Menten who assume the slowest step is the decomposition of the intermediate complex (Ashmore, 1963). Thus $k_3 \ll k_2$.

$$V_o = \frac{k_3[E]_o}{1 + \frac{k_2}{k_1[S]_o}} = \frac{V_{max}}{1 + \frac{K_m}{[S]_o}}$$

The Michaelis constant, K_m, is the dissociation constant of the enzyme-substrate complex. From steady-state rates it is difficult to determine if the measured rate constant is K or K_m.

Kistiakowsky and Thompson (1956) found the Michaelis constant of urease to be independent of ionic strength. The first step involves the reaction of an uncharged urea molecule with the enzyme so the rate constants for the forward and reverse reations, k_1 and k_2, will be independent of ionic strength. Thus only k_3 depends on ionic strength so it must have little or no effect on the overall rate constant and must be small with respect to k_2. Thus k_3 is much less than k_2 and the Michaelis constant of urease is the form $\frac{k_2}{k_1}$.

The mechanism of the urease-catalyzed hydrolysis of urea is not known in detail but it has been established that carbamic acid is an intermediate in the reaction (Wang and Tarr, 1955). Investigation of the kinetics has been complicated by the most commonly used buffers affecting the reaction. Wall and Laidler (1953a) were able to show that trishydroxymethylaminomethane sulphate had no effect on the enzyme. The reaction rates agreed with the Michaelis-Menten equation at low to intermediate substrate concentrations but decreased at higher concentrations due to substrate inhibition.

Contrary to the findings of Wall and Laidler (1953b), Kistiakowsky et al (1952) found the activity of urease solutions to vary linearly with urease concentration. At very low substate concentrations the initial rates deviate slightly from the Michaelis-Menten equation. It has been suggested that urease either possesses two types of active sites with differing Michaelis-Menten parameters or pairs of identical active sites which interact when one of the sites combines with urea (Kistiakowsky and Rosenberg, 1952).

The overall kinetics of urea hydrolysis is fairly simple but recent work on kinetic isotope effects (Lynn and Yankwich, 1964) indicates that the exact mechanism is very complex.

Reactions of Urea in the Soil

When urea is added to the soil it usually hydrolyzes very rapidly. The ammonium ions produced are oxidized to nitrate by soil microorganisms. Ammonia is first converted to nitrite, mostly by

Nitrosomonas spp., and the oxidation of nitrite to nitrate is carried out by Nitrobacter spp. (Alexander, 1961).

If hydrolysis is slow, urea can be leached, particularly in sandy soils; however, in some soils it does not leach easily and appears to be partially adsorbed by the soil. Urea adsorption in soil is primarily a chemisorption phenomenon with the organic matter fraction being responsible for most of the adsorption and little or no adsorption by the clay mineral fraction (Chin and Kroontje, 1962). The formation of salts with the acidic groupings of soil organic matter by urea is the most likely mechanism for the adsorption (Broadbent and Lewis, 1964). Farmer and Ahlrichs (1969), prepared deuterated urea complexes of calcium, nickel and aluminum montmorillonites. Using infrared spectroscopy they determined the bonding between the urea and the clay to occur at the carbonyl group of the urea. They suggested that in the case of a transition metal cation the carbonyl group coordinates with an empty d-orbital of the interlayer cation.

Due to the alkalinity of the final reaction product, ammonium carbonate, the hydrolysis of urea raises the soil pH. At pH values greater than eight the partial pressure of ammonia gas in equilibrium with aqueous ammonium ions is quite high, so under certain conditions appreciable quantities of ammonia could be volatilized (Court, Stephen and Waid, 1964a).

Occurrence of Urease Activity in Soil

The hydrolysis of urea in soil is entirely biochemical in nature as the chemical hydrolysis in soil is negligible at normal temperatures (Chin and Kroontje, 1963). The hydrolysis can be carried

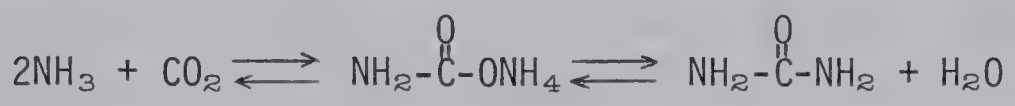
out by many soil microorganisms, but can also occur in the absence of viable microorganisms because soil sterilized by either toluene (Stojanovic, 1959) or gamma radiation (Roberge and Knowles, 1968) showed little change in hydrolytic activity.

Urease activities of soils are greatest during the summer months when temperatures and the biological activity are highest (Stojanovic, 1959). Soils high in organic matter tend to have greater urease activities, and the addition of organic matter to soil increases the urease activity (Conrad, 1942). McGarity and Myers (1967) found a positive correlation between organic carbon content and the urease activity. Thus urease activity is generally related to the microbial activity in the soil.

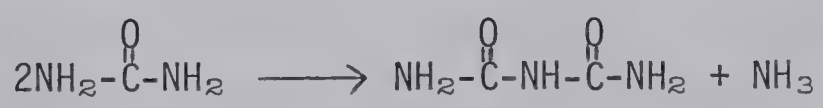
It is likely that 79% to 89% of the hydrolytic activity can be attributed to urease that has been released by dead microorganisms and then complexed by soil colloids (Paulson and Kurtz, 1969). Pinck and Allison (1961) were able to show that urease can be completely adsorbed by hydrogen montmorillonite. The clay-urease suspension they obtained still possessed urease activity. They postulated that urease is adsorbed at the cation exchange sites, is inactive in the adsorbed form, and for the suspension to show activity the urease must be replaced by a cation and released into solution. They demonstrated that urea itself could effect the release of urease from the clay-urease complex and produce free urease in solution, but were not able to show the mechanism of this phenomenon.

Efficiency of Urea as a Fertilizer

Urea is commercially manufactured by reacting ammonia with carbon dioxide at elevated pressure and temperature to form ammonium carbamate which decomposes to urea and water:



This reaction is carried out at a temperature between 175°C and 210°C and a pressure between 170 atm. and 400 atm. (Sauchelli, 1964). The urea solution is concentrated by evaporation and then it is either melted and prilled or crystallized. In either of these processes some biuret may be formed by heat-induced dimerization of the urea:



In some instances urea has been shown to be inferior to other nitrogenous fertilizers. Court et al (1963) found urea to be a less effective fertilizer than ammonium nitrate for maize. Various theories have been postulated to explain the difficulties associated with urea. Toxicity could arise from the isomerization of urea to ammonium cyanate but concentrations likely to occur in the soil from the addition of urea would have no effect on plant growth (Low and Piper, 1961). Carbamic acid which might be phytotoxic is an intermediate in the enzyme-catalyzed hydrolysis of urea. However it is a short-lived intermediate and would not accumulate in appreciable quantities. Urea itself may be absorbed by plant roots (Hutchinson and Miller, 1912;

Wallace and Ashcroft, 1956) but it is not known if it can be absorbed in sufficient quantities to be harmful.

Manufactured urea always contains small amounts of biuret. Urea containing more than 2.5% biuret reduces germination in wheat (Smika and Smith, 1957) and when it contains more than 2% is harmful to corn seedlings (Wilkinson and Ohlrogge, 1960). Urea produced in recent years has a biuret content less than 1% which will not adversely affect crop growth. It is not likely that biuret will accumulate in the soil because some soil microorganisms are able to utilize it as a nitrogen source (Jensen and Schroder, 1965). The possibility that soil microorganisms can polymerize urea to biuret has not been investigated.

Most of the situations where urea has been found to be a less effective nitrogen source can be attributed to high concentrations of ammonia resulting from a rise in pH and high concentrations of ammonium ions. The ammonia produced at high pH is toxic to plants and would also result in the loss of nitrogen by volatilization as ammonia.

It has been suggested (Court, Stephen and Waid, 1964a, 1964b) that high pH values and ammonium ion concentrations would prevent the oxidation of nitrite to nitrate by Nitrobacter spp. so toxicity could arise from the accumulation of both ammonia and nitrite. The nitrite ion is not toxic to plants at neutral or alkaline pH (Duisberg and Buehrer, 1954), but as nitrification proceeds and the pH drops, the accumulated nitrite ions could become toxic to plant growth (Court, Stephen and Waid, 1964a).

Vines and Wedding (1960) studied the mechanism of ammonia toxicity in plants and found ammonia gas to be responsible for the toxicity problem. Ammonia interferes with the respiration of plant cells by inhibiting the oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). The pH affects the toxicity only because the partial pressure of ammonia increases at higher pH values.

Overrein and Moe (1967) determined volatilization losses of nitrogen from urea to be as high as 75% under severe conditions. There is less volatilization loss in soils of high cation exchange capacity because the excess ammonium ions are adsorbed on the exchange complex (Gasser, 1964).

Methods of Altering the Rate of Urea Hydrolysis in Soil

If the rate of urea hydrolysis could be retarded both the toxicity and volatilization of ammonia would be reduced. This could be done by several methods such as altering the urease activity of the soil or by using slow release fertilizers. A slow release urea fertilizer can be made either by coating the urea pellets with an inert substance or by making a urea-formaldehyde polymer.

Moe (1967) altered the activity of the soil by the addition of urease and decomposing mulch to increase the hydrolysis rate and the addition of a urease inhibitor, p-chloromercuribenzoate, to decrease the rate. There was no significant difference in total volatile ammonia losses between the various treatments. The total volatilization was not reduced by p-chloromercuribenzoate because it also inhibited nitrification so a high concentration of ammonium ions

would build up eventually. This problem could be overcome by using an inhibitor specific for urease. Acetohydroxamic acid inhibits urease specifically and has been used to suppress bovine rumen urease activity (Jones, 1968).

Conclusions

The enzymatic hydrolysis of urea can be explained by the Michaelis-Menten equation, with some deviation from it at very low concentrations. Carbamic acid is known to be an intermediate in this reaction but the exact mechanism has not been determined.

Sumner et al (1938) determined a molecular weight of 483,000 for urease, but more recently a molecular weight of 487,000 has been measured for urease. Polymers of urease have been shown to exist, and the enzyme also has structural subunits with molecular weights of 83,300. It is very sensitive to denaturation by heavy metals but can be protected by various colloidal materials.

When urea is added to the soil it is normally hydrolyzed very rapidly. The ammonium ions produced can then be oxidized to nitrate by the nitrifying bacteria in the soil. If hydrolysis is slow urea can be leached fairly easily as it only has a slight affinity for soil colloids. Many soil microorganisms are capable of hydrolyzing urea but most of the activity in soil is due to urease complexed by soil colloids. Greater urease activities are usually associated with soils high in organic matter content.

Urea has been shown to be less efficient than other nitrogenous fertilizers primarily due to ammonia toxicity and the loss of nitrogen

by volatilization as ammonia. The difficulties with urea are promoted by: rapid hydrolysis, high pH, and low soil cation exchange capacity.

The hydrolysis rate of urea in soil can be altered in several ways. It can be increased by enhancing the urease activity of the soil and can be decreased by either using slow release fertilizers or adding urease inhibitors to the soil.

III. MATERIALS AND METHODS

Materials:

The soil samples used in this investigation were obtained from three sites. Site I is a virgin Gray Wooded soil located in the Cooking Lake region (NW7-51-21-W4) and Site II is located on a cultivated Gray Wooded Soil 100 yards north of Site I. The soil at Site III is a Chernozem (Malmo clay loam) and is located on the University farm (NE24-51-25-W4) at Ellerslie. This location was seeded to grass during 1964-67 and was fallow from 1967 to 1969.

The profile description of Site I is given in Appendix I, the data from mechanical and chemical analyses in Appendix II, and all the soil samples with the sampling dates and other pertinent information are listed in Appendix III.

The compounds used in the inhibition studies were obtained from the Research Council of Alberta. These compounds and their structures are given in Appendix IV.

Methods:

(a) Routine analyses:

The mechanical analyses of the soil samples were carried out by the pipette method (Kilmer and Alexander, 1949). pH values were determined on a saturated soil paste as described by Doughty (1941) using a Beckman model zeromatic pH meter. Cation exchange capacity was determined by leaching the samples with normal ammonium acetate, extracting the adsorbed ammonium with normal NaCl and distilling the extract by the magnesium oxide method (A.O.A.C. 1955). Total carbon

content was determined by the Leco dry combustion method. Each sample was placed in a Leco induction furnace, oxidized, and the carbon dioxide evolved was measured manometrically.

(b) Measurement of the hydrolysis rate

The hydrolysis rate of urea in soil was determined by a method based on that employed by Simpson and Melsted (1963). The method of Simpson and Melsted (1963) was found to be unsatisfactory and was modified. The samples were incubated in 200 ml Erlenmeyer flasks. To each flash was added 25 g of air-dry soil, 5 ml of urea solution, and enough distilled water to produce the moisture level desired for that particular experiment. The blanks contained the same quantities of soil and water as the samples. The flasks were sealed with parafilm and placed in a constant temperature incubator. At various time intervals three samples and one blank were removed from the incubator and the urea was extracted and analyzed.

In order to extract urea from the soil the following was added to each sample: 0.2 g $CaCl_2$, 0.2 g Norit-A decolorizing carbon, 5 ml of 1% $HgCl_2$ solution, and enough distilled water to dilute to a total water volume of 100 ml. The samples were shaken for 20 minutes and filtered through Whatman #1 filter paper. To 15 ml of the filtrate was added 10 ml of urea color reagent (Watt and Chrisp, 1954). The color reagent consists of: 10 ml concentrated HCl, 2 g p-dimethylaminobenzaldelyde, and 100 ml of 95% ethyl alcohol. The samples were allowed to stand for 10 minutes so the color would stabilize, and then the percent transmittance was measured on a Bausch

and Lomb Spectronic 20 at 430 mμ. The standard curve was prepared using Fisher Analytical Grade Urea (ACS Certified). The standard curve is given in Appendix V.

A refrigerator was used to incubate the samples at 2°C and 7°C. A Fisher Low Temperature Incubator was used to incubate samples for the range 13°C to 30°C, and a Fisher Senior Isotemp Oven for runs above 30°C. Approximately one hour before each experiment was begun the distilled water, urea, and soil samples were stored separately at the temperature of the experiment. This was particularly necessary for the experiments carried out at temperatures differing significantly from room temperature.

Unless otherwise stated the experiments discussed in this thesis were carried out at 25°C, 24% moisture, and an initial substrate concentration of 200 ppm urea N. To obtain an initial urea concentration of 200 ppm urea N in the soil it was necessary to add a 5 ml aliquot of a 2140 ppm urea solution to 25 g of soil. A moisture concentration of 24% was used in many of the experiments since it is equivalent to the 1/3 atm. moisture tension for the surface horizon of Site III.

(c) Sampling procedure

Five or six subsamples were taken from each plot, mixed and a composite sample used. After the soil samples had been obtained they were air-dried for three days, ground to pass a 12 mesh sieve and stored in plastic bags.

(d) Urea-treated soil

An experiment to determine the effect of urea on soil urease activity was carried out at Site III. On June 11, 1969, urea was

applied to three plots at the following levels: 0, 100, and 800 lb. N/acre. At later intervals samples were taken from these plots and the hydrolysis rate was measured under the following conditions: 25°C, 24% moisture, and 200 ppm nitrogen as urea. The urease activities in these samples were calculated on the basis of an 8 hr. incubation period.

(e) Hydrolysis rates in various soils

The urease activity was measured in a cultivated Malmo clay loam (sample 7), a cultivated Gray Wooded soil (sample 16) and various horizons from a Brunisolic Gray Wooded soil (samples 8-15). The following conditions were used to measure the urease activities of these samples: 25°C, 50% moisture, and an initial substrate concentration of 200 ppm nitrogen as urea.

(f) Urease inhibitors

The effect of various urease inhibitors on the hydrolysis rate of urea in soil was determined in triplicate under the following conditions: 25°C, 24% moisture, and an initial substrate concentration of 200 ppm nitrogen as urea. To each of the control samples 5 ml of urea solution was added; and 5 ml of solution containing both urea and inhibitor was added to each of the treated samples. After the samples had been incubated for 8 hours the urea was extracted and analyzed. Then the quantities of urea hydrolyzed in the control and the inhibitor treated samples were used to calculate the percent reduction in hydrolysis for the inhibitor treated samples.

IV. RESULTS AND DISCUSSION

Development of Extraction Technique

The extraction procedure used by Simpson and Melsted (1963) is unsatisfactory because it gives incomplete recovery of added urea. Simpson and Melsted extracted urea from 25 g samples of soil using 50 ml of water, 0.15 g CaO, and 0.15 g Darco G-60. The samples were then shaken, centrifuged and decanted.

In order to determine the best extraction procedure, 25 g soil samples were autoclaved for 20 minutes, an aliquot of urea added, and the urea was extracted by several methods and analyzed. Varying quantities of $CaCl_2$, Norit-A and water were added to the samples which were then shaken for 20 minutes and filtered through Whatman #1 filter paper. Aliquots of the filtrates were analyzed for urea.

The data in Table I indicate that both soil and decolorizing carbon will adsorb urea. The recovery can be improved by adding either more $CaCl_2$ or water. Since $CaCl_2$ has no effect on the transmittance of the analytical reagent as measured with spectrophotometer the measured increase in recovery is a real effect. Thus $CaCl_2$ must be desorbing urea from both the soil and the decolorizing carbon. An additional experiment with unsterilized soil using $HgCl_2$ in the extraction procedure gave 99% recovery of 200 ppm urea nitrogen.

The extraction procedure that was finally adopted involved the addition of the following: 5 ml of 1% $HgCl_2$, 0.2 g $CaCl_2$, 0.2 g Norit-A decolorizing carbon and sufficient distilled water to produce a total water volume of 100 ml. Mercuric chloride was added to kill

TABLE 1

Effect of Various Quantities of Extractants on Urea Recovery

Urea N added, ppm	Norit-A added, g	$CaCl_2$ added, g	Water added, ml	% Recovery
200	0.2	5.0	100	100
200	0.2	5.0	50	99
200	0.2	0.2	100	98
200	0.2	0.2	50	92
200	1.0	0.2	100	88
400	0.2	0.2	100	99
400	0.2	0.2	50	86

the bacteria and inactivate soil urease. Norit-A was added to clarify the filtrate in order to reduce interference in the spectrophotometric measurement. Calcium chloride aids filtration by flocculating the soil and also desorbs urea from the soil.

Effect of Concentration on the Hydrolysis of Urea in Soil

Simpson and Melsted (1963) studied the hydrolysis of urea in several soils at initial urea concentrations of 200 and 400 ppm N. The hydrolysis rate was zero-order with respect to urea in most of the soils they studied. Paulson and Kurtz (1970) determined the Michaelis constant of soil urease and found it to be somewhat higher than bacterial urease. They suggested that urease adsorbed on soil colloids has a higher Michaelis constant than free urease. The Michaelis constant of any enzyme depends on the environment of that particular enzyme. The following variables have a significant effect on the Michaelis constant of an enzyme: temperature, pH, inhibitors, activators, and the presence of any material that may react with or complex the enzyme. The true Michaelis constant is the one measured in the absence of any interferences at the optimum pH (Waley, 1953).

The hydrolysis of urea in a Malmo clay loam was neither zero order nor first order (figures 1 and 2). However there was a linear increase in rate with concentration (figure 3) which is characteristic of first order reactions. The average rate for the first half of the reaction was arbitrarily chosen as the reaction rate for most of the experiments described in this thesis. Although initial rates are preferable for kinetic studies, there was too much variation in the

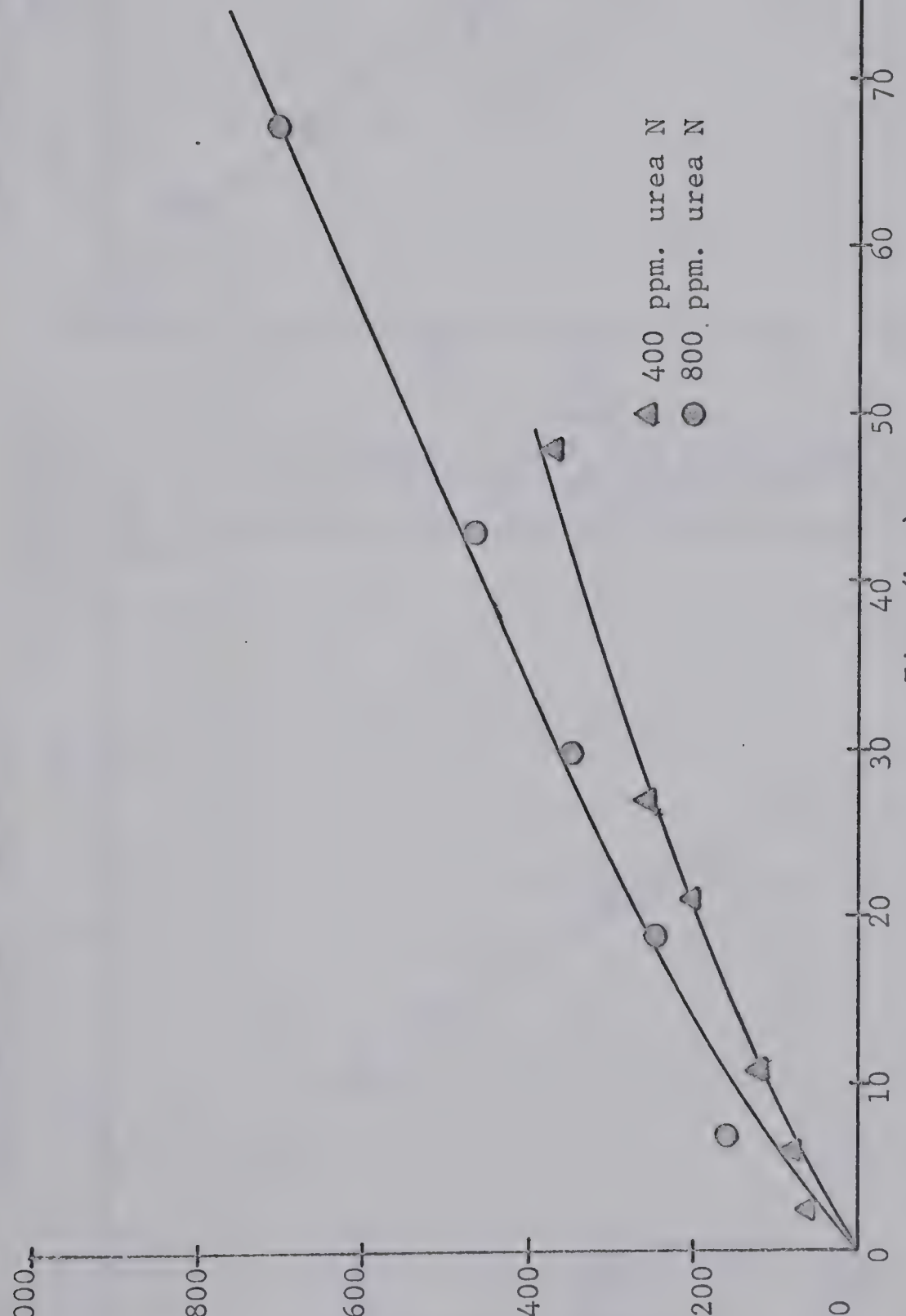


Figure 1. The hydrolysis rates at initial urea concentrations of 400 and 800 ppm. N. in a Malmo clay loam. Experimental conditions: 25°C and 24% moisture. Soil sample #1 used; sample stored 1 week before use.

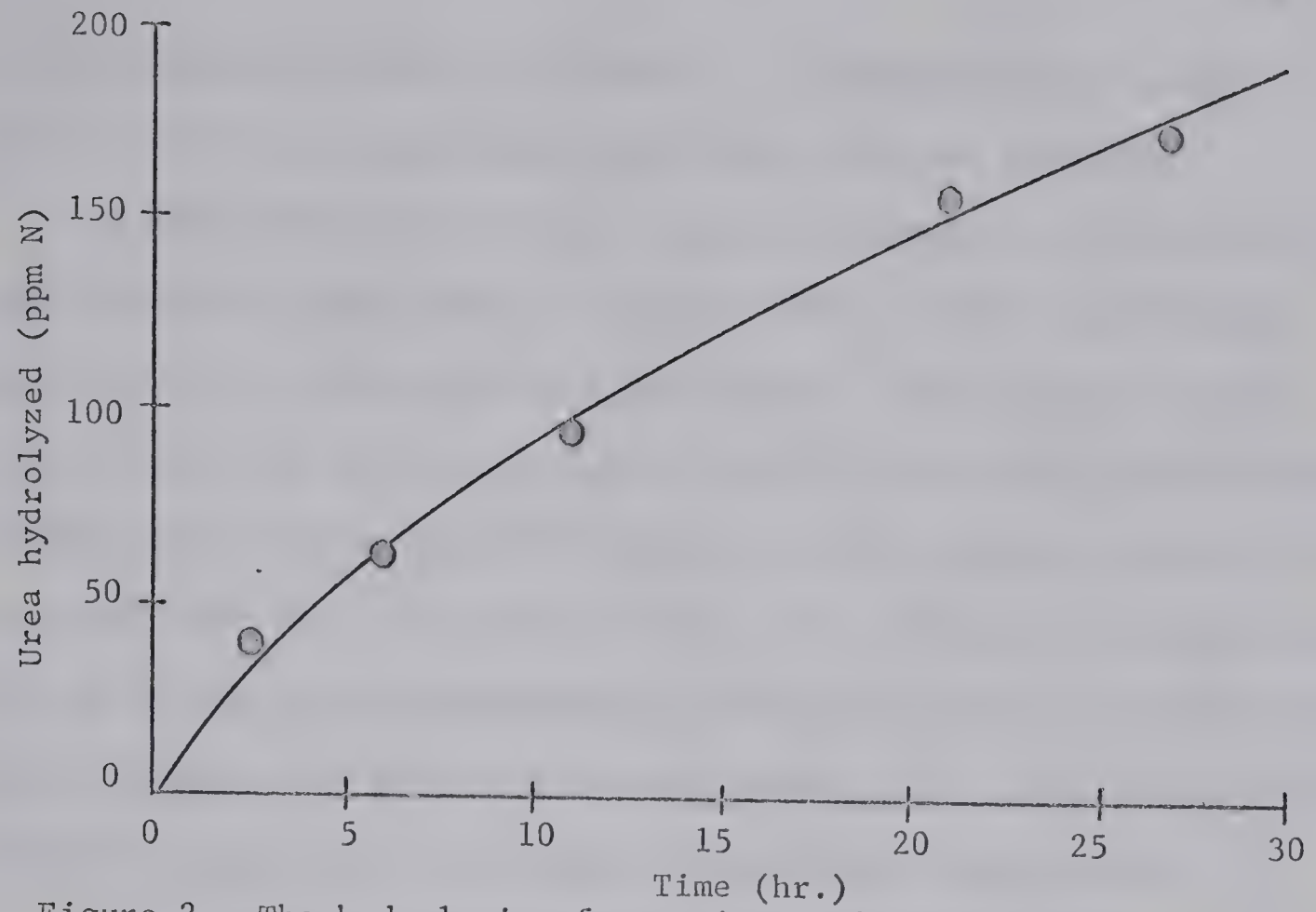


Figure 2. The hydrolysis of urea in a Malmo clay loam at an initial substrate concentration of 200 ppm urea N. Experimental conditions: 25°C and 24% moisture. Soil sample #1 used; sample stored 1 week before use.

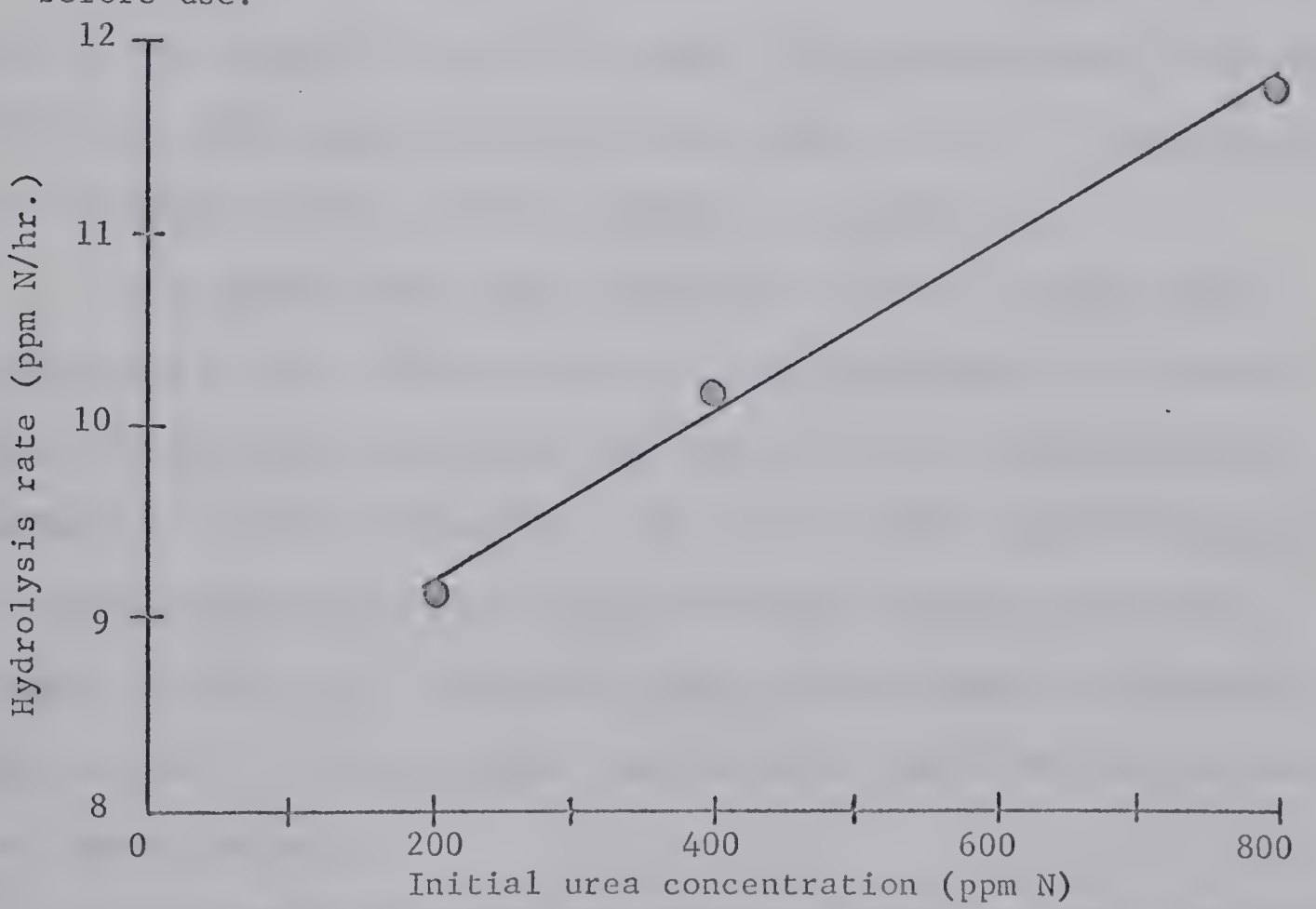


Figure 3. The effect of the initial urea concentration on the hydrolysis of urea in a Malmo clay loam. Experimental conditions: 25°C and 24% moisture. Soil sample #1 used; sample stored 1 week before use.

initial points for them to be useful. A Lineweaver-Burke graph was not plotted because the initial rates were not available.

The initial soil pH was 6.4 which increased to 6.5 after 200 ppm urea N had hydrolyzed, to 6.8 after 400 ppm urea N hydrolyzed, and to 7.5 after 800 ppm urea N hydrolyzed. The greatest increase was 0.4 pH units in the experiment with an initial urea concentration of 800 ppm N because only the first half of the reaction was used to calculate the rate. The effect of pH on the rate would be negligible for an initial urea concentration of 200 ppm but would be significant for 800 ppm N. To clarify the concentration effect the initial rates should be determined for a number of substrate concentrations.

Effect of Storage Time on Urease Activity

Soil urease activity is affected by the way the soil is treated and by the length of time it is stored. The activity tends to increase initially after sampling (McGarity and Myers, 1967). Air drying of the soil produces an even greater increase in activity.

The average rate during the first half of the reaction was calculated as the rate for each particular experiment. The urease activity increases appreciably for about 75 days and then remains relatively constant (figure 4). The initial rise is most likely due to microorganisms dying and lysing which would release additional urease into the soil. Since the urease activity does not decrease when the soil is stored urease must be quite stable for long periods of time in dry soil.

Since storage time has a significant effect on urease activity comparisons can only be made between samples that have been stored for similar lengths of time.

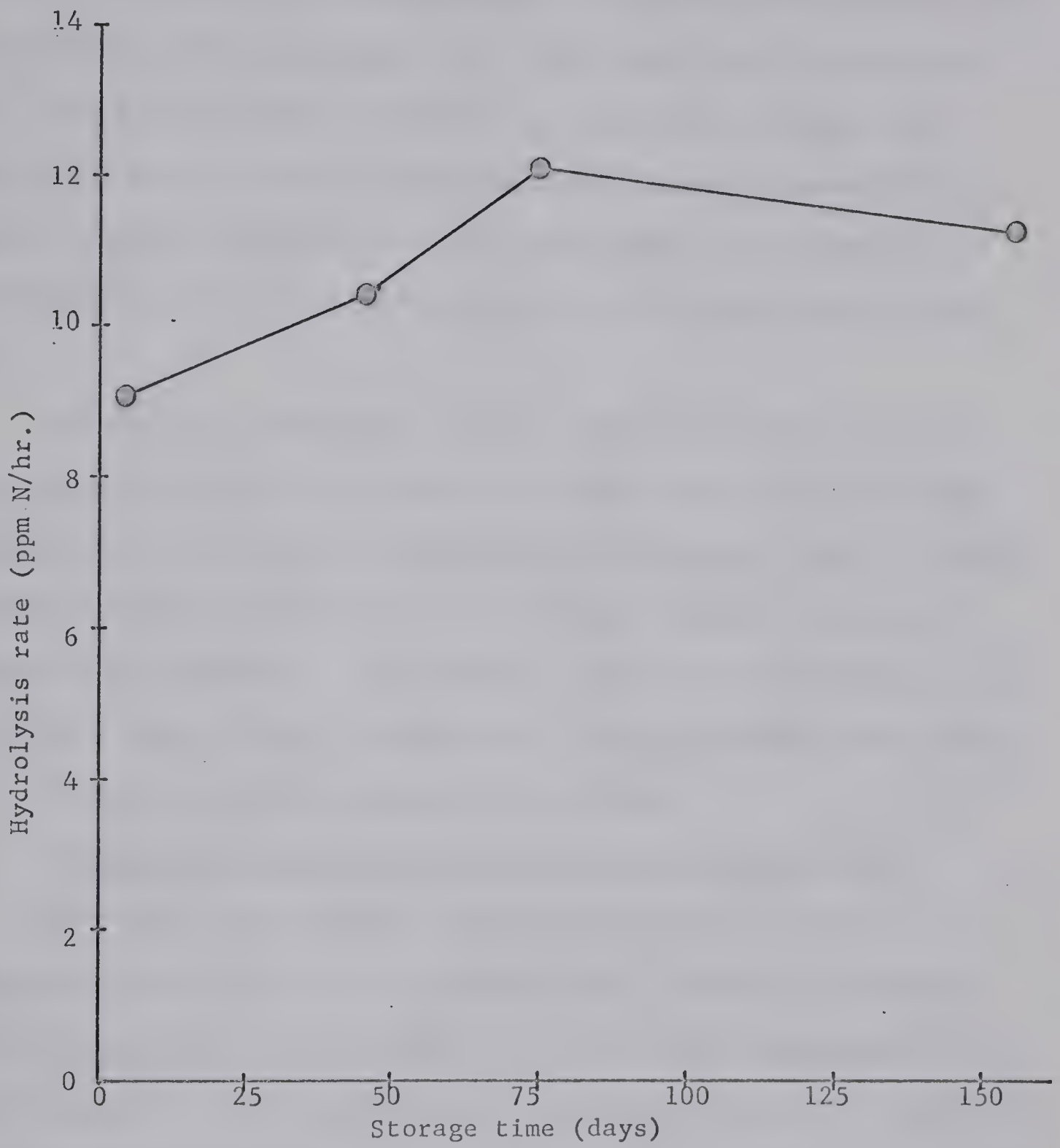


Figure 4. The effect of storage time on soil urease activity in a Malmo clay loam. Urease activity measured using the following conditions: 25°C, 24% moisture, and an initial substrate concentration of 200 ppm urea N. Soil sample #1 used.

Effect of Moisture on the Hydrolysis of Urea in Soil

The rate at which urea diffuses to the urease molecule may have some effect on the hydrolysis rate. Both temperature and moisture will affect the diffusion rate, so it is necessary to study the hydrolysis rate at various temperatures and moisture concentrations. Moisture levels below 24% were not investigated. At low moisture concentrations the urea solution may not be in contact with all the soil.

At 25°C the hydrolysis rate was highest for 50% moisture and the rates were essentially equal for moisture levels of 24 and 100% (figure 6). The effect is reversed at 13°C since the rate is slightly higher at 24% moisture (figure 5). Moisture concentration has no effect on the hydrolysis rate at 40°C. The effect of moisture on the hydrolysis rate of urea in a Malmo clay loam at moisture levels above 1/3 atm. moisture tension appears to be slight.

Effect of Temperature on the Hydrolysis of Urea in Soil

The rate of all enzyme catalyzed reactions increases as the temperature rises until at high temperatures it begins to decrease due to inactivation of the enzyme. The temperature dependence of the rate constant at lower temperatures can be described by the Arrhenius equation (Barrow, 1961):

$$K = Ae^{-E_a/RT}$$

K = rate constant
T = temperature in °K
E_a = activation energy
R = gas constant
A = pre-exponential factor

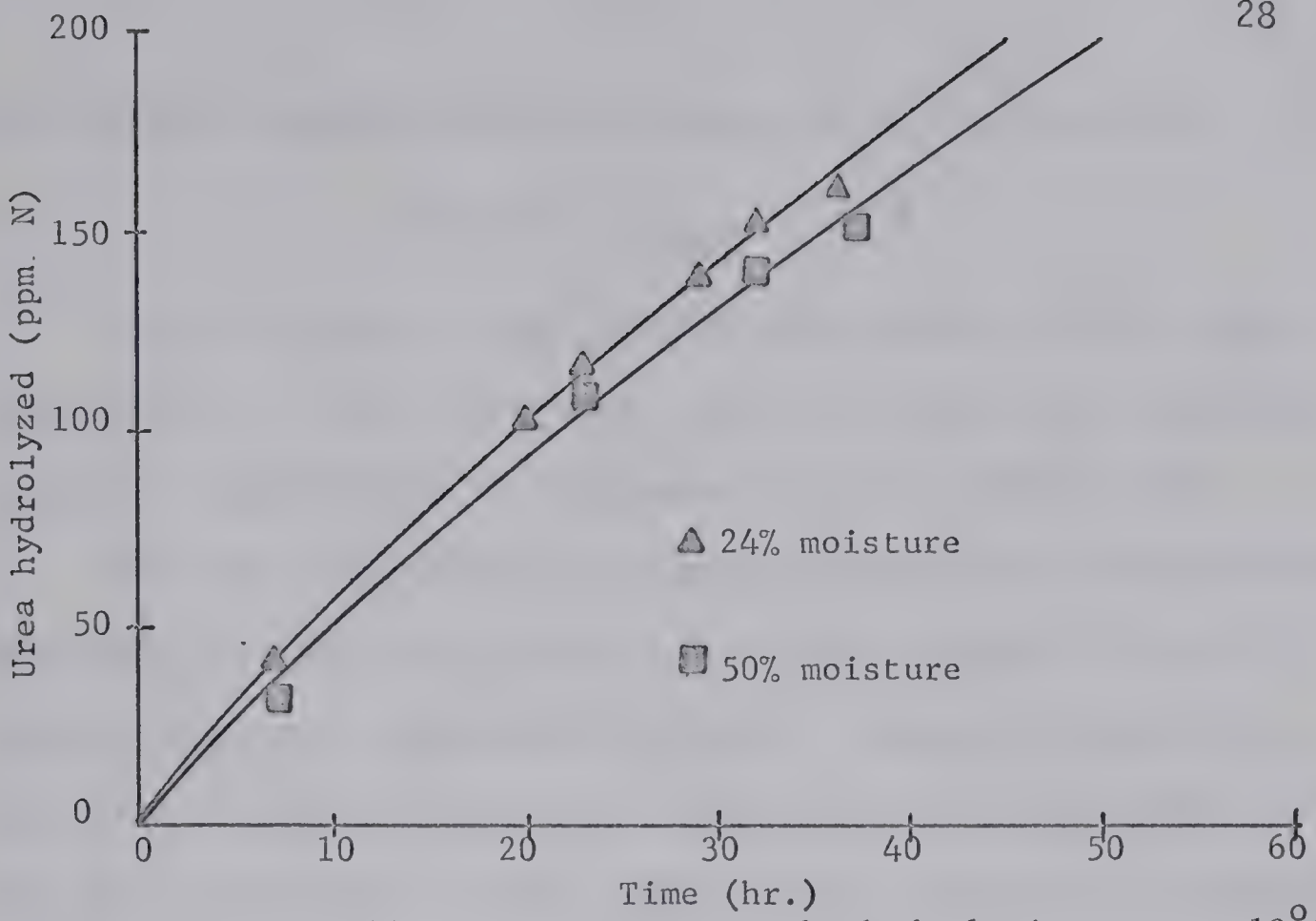


Figure 5. The effect of moisture on the hydrolysis rate at 13°C in a Malmo clay loam. Initial substrate concentration of 200 ppm urea N. Soil sample #1 used; samples stored 2 weeks before use.

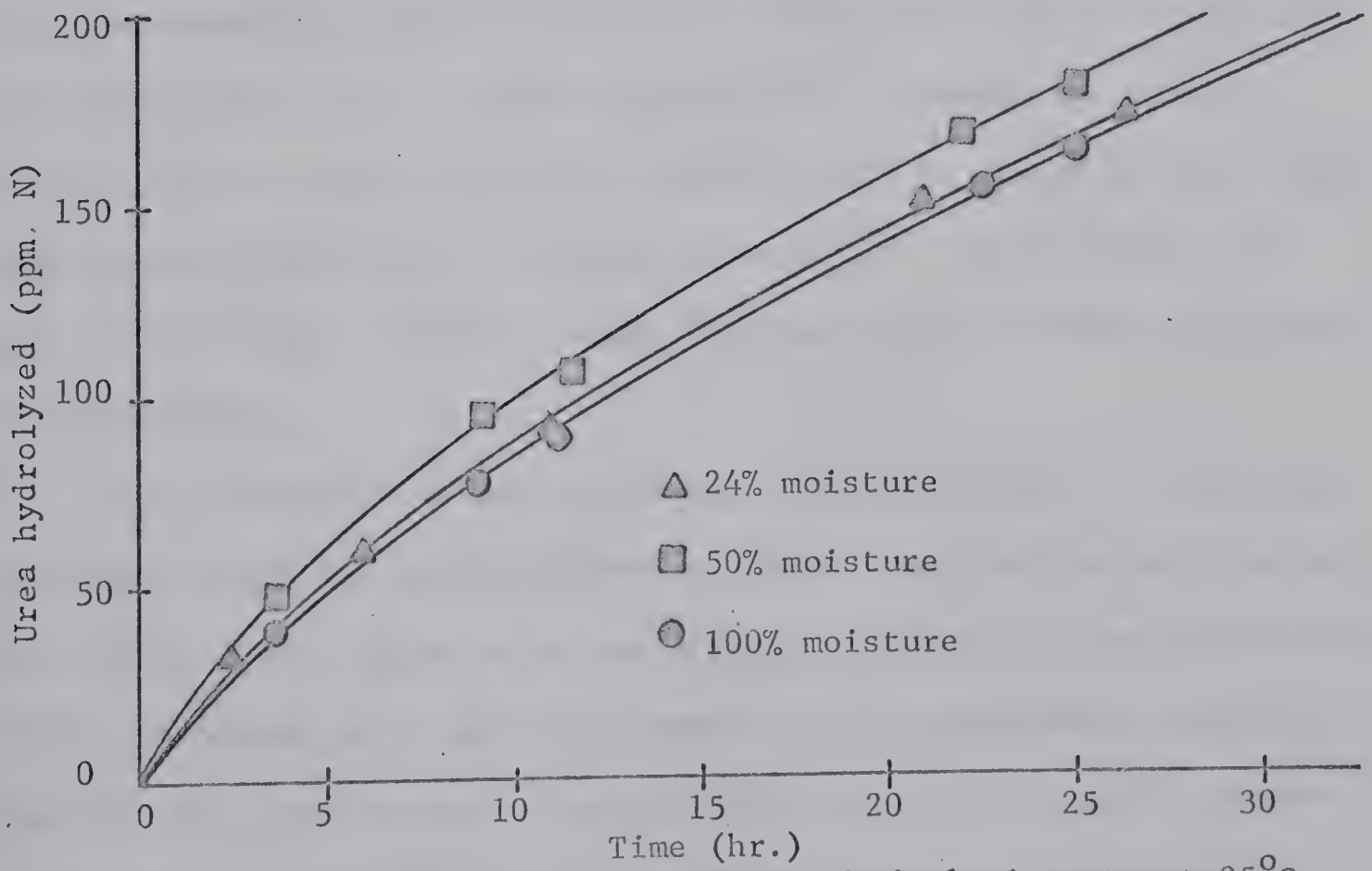


Figure 6. The effect of moisture on the hydrolysis rate at 25°C. in a Malmo clay loam. Initial substrate concentration of 200 ppm urea N. Soil sample #1 used; samples stored 2 weeks before use.

The Arrhenius equation can also be expressed in the log form:

$$\log K = - \frac{E_a}{2.303RT} + \log A$$

A plot of log K or log (initial rate) versus 1/T will give a straight line of slope $-E_a/2.303R$. Since R is known, the activation energy of a reaction can be determined from the Arrhenius plot.

The activation energy of an enzyme depends upon the experimental conditions, and the most important variables affecting it are ionic strength, substrate concentration and pH. Jack bean urease has an activation energy of 6.8 kcal/mole at pH 7.13 and 8.5 kcal/mole at pH 8.00 (Wall and Laidler, 1953a). Under certain conditions discontinuities occur in the temperature plot (Sizer, 1943). Sizer found that in the presence of mild reductants the activation energy is 8.7 kcal/mole and in the presence of mild oxidants is 11.7 kcal/mole. At intermediate oxidation-reduction potentials the activation energy is 8.7 kcal/mole above 22°C and is 11.7 kcal/mole below 22°C. Although the effect is disputed, Sizer suggests that the discontinuity is caused by the enzyme changing configuration as the temperature rises. Kistiakowsky and Lumry (1949) found a gradual change in slope of the Arrhenius plot with no discontinuity.

Soil urease is of both plant and microbial origin. It has been found that bacterial urease differs slightly from plant urease (Larson and Kallio, 1954). Baterial urease (Bacillus pasteurii) has an activity of 190,000 Sumner units per gram compared to 133,000 Sumner units per gram for jack bean urease. The activation energy of bacterial urease is 9.9 kcal/mole below 25°C and 4.4 kcal/mole above that temperature.

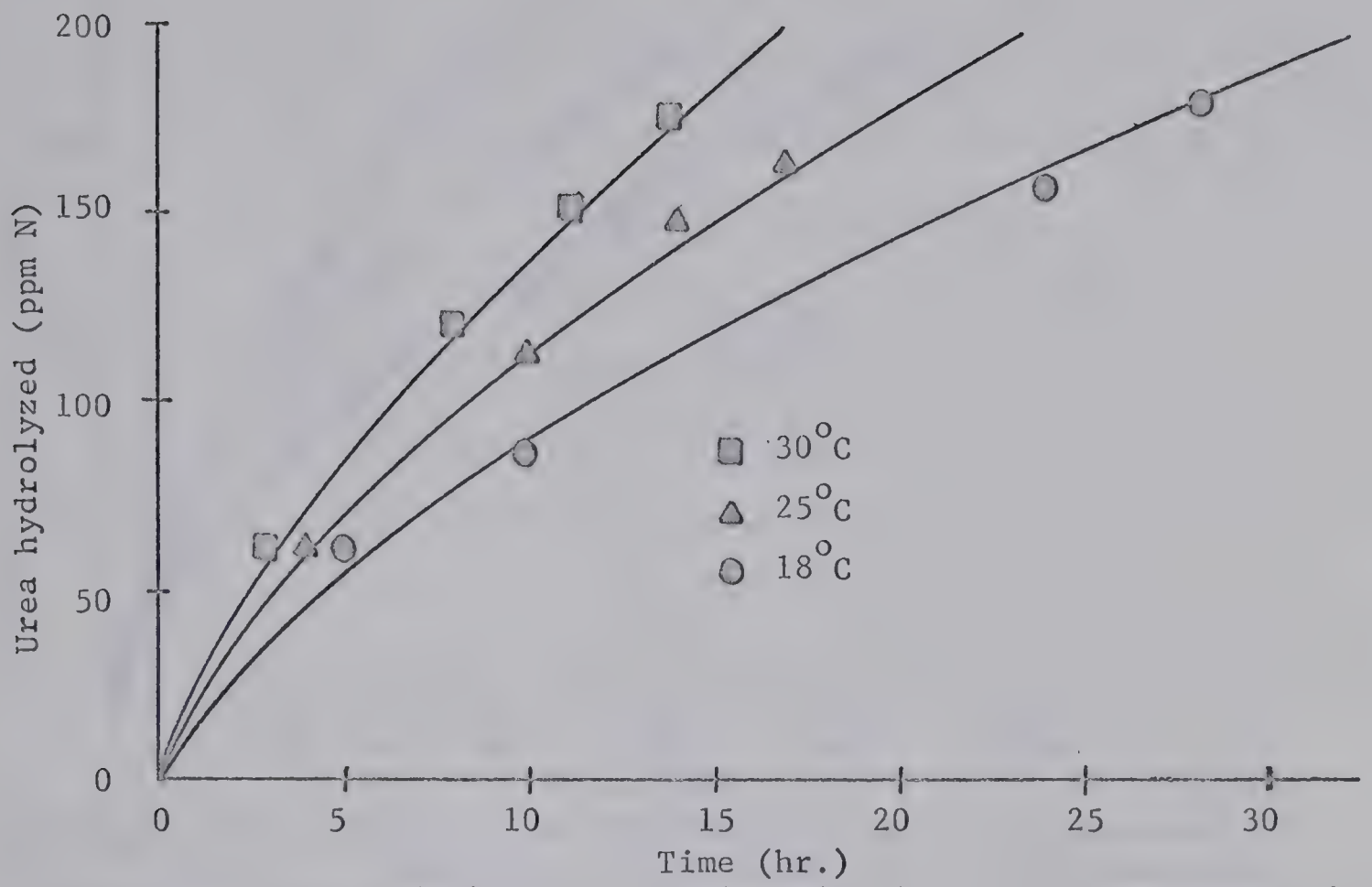


Figure 7. Urea hydrolysis in a Malmo clay loam at temperatures of 18°C, 25°C, and 30°C. Experimental conditions: 24% moisture and an initial substrate concentration of 200 ppm urea N. Soil sample #1 used; sample stored 3 weeks before use.

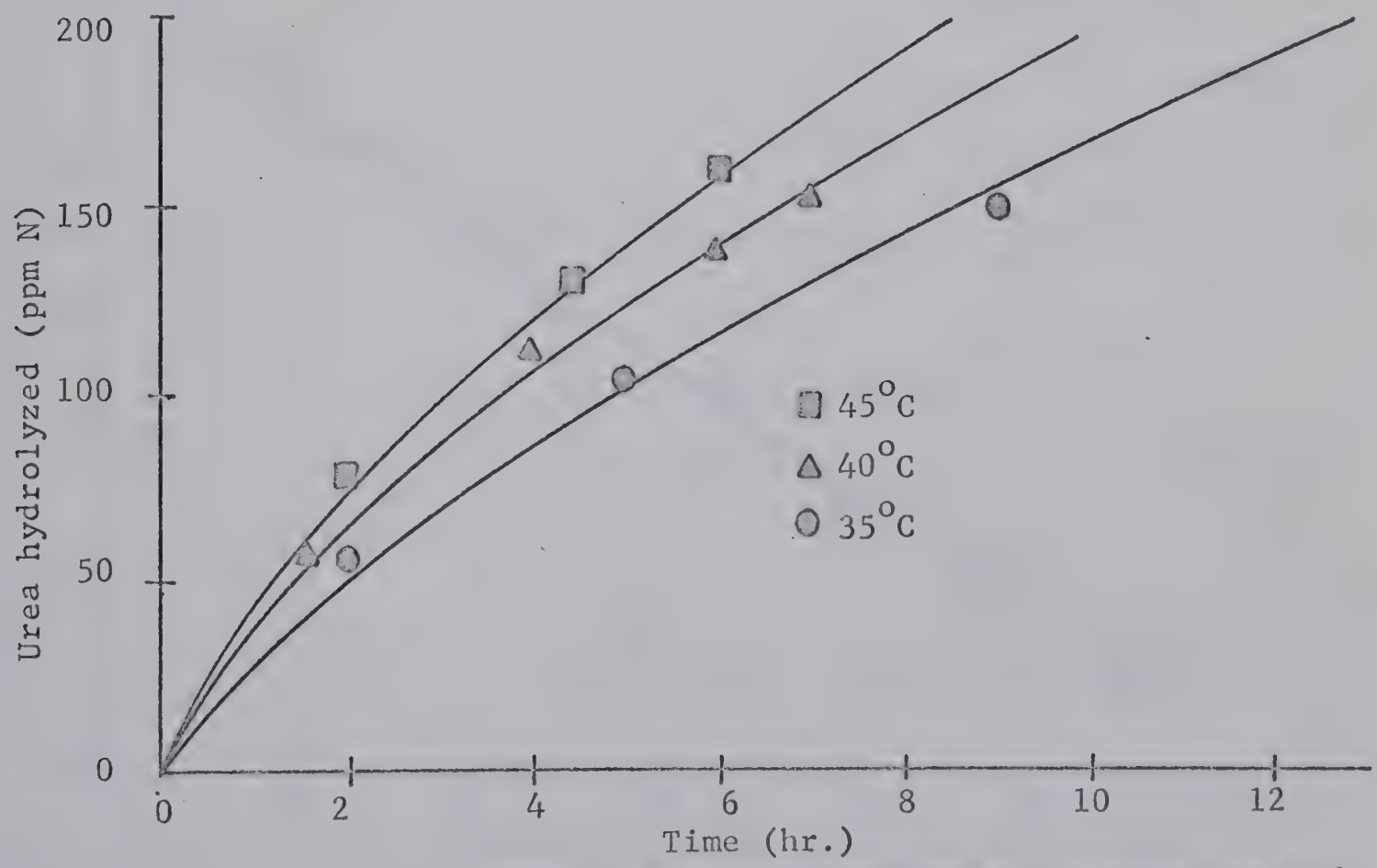


Figure 8. Urea hydrolysis in a Malmo clay loam at temperatures of 35°C, 40°C, and 45°C. Experimental conditions: 24% moisture and an initial substrate concentration of 200 ppm urea N. Soil sample #1 used; sample stored 3 weeks before use.

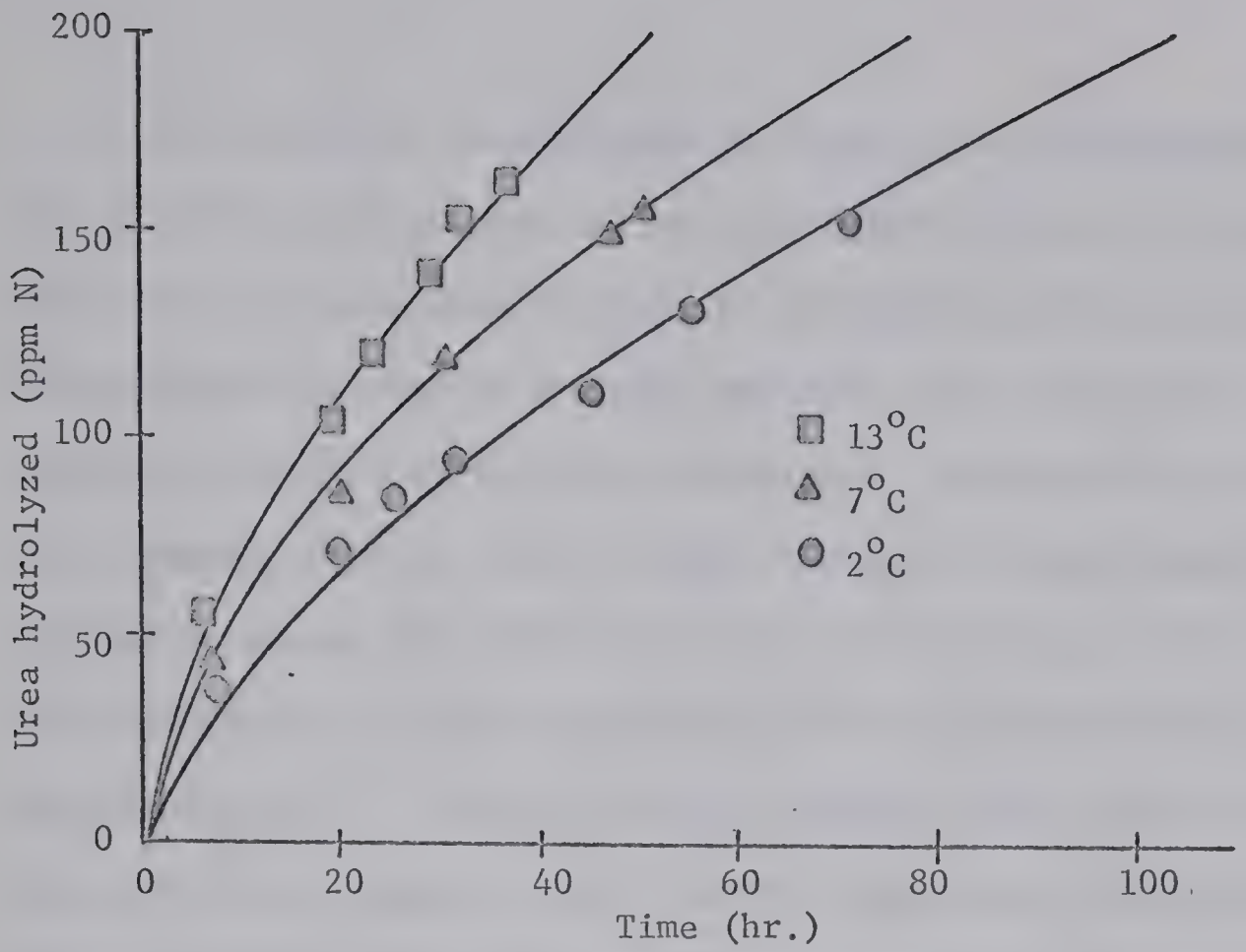


Figure 9. Urea hydrolysis in a Malmo clay loam at temperatures of 2°C, 7°C and 13°C. Experimental conditions: 24% moisture and an initial substrate concentration of 200 ppm urea N. Soil sample #1 used; samples stored 3 weeks before use.

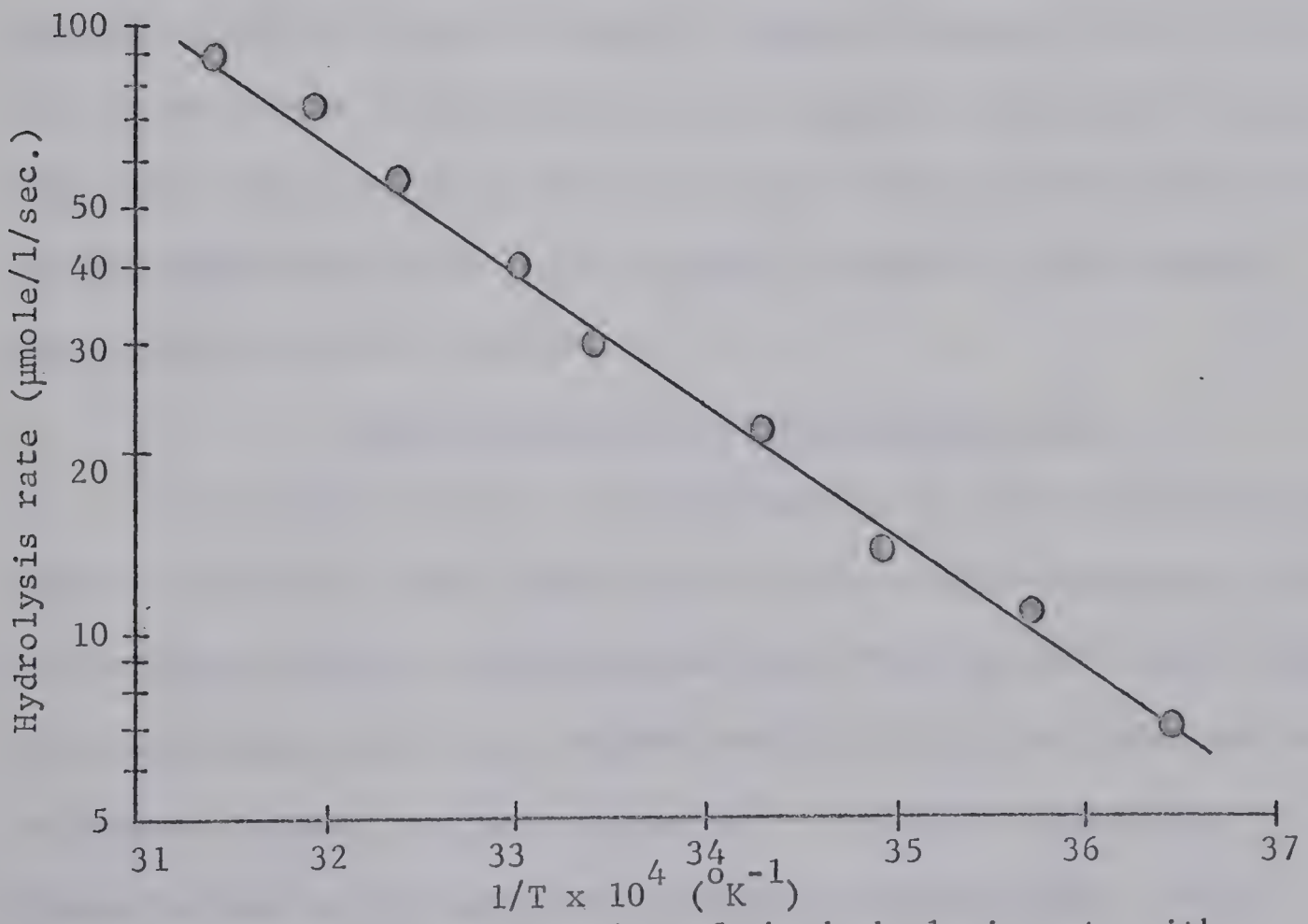


Figure 10. Variation of the log of the hydrolysis rate with inverse temperature in a Malmo clay loam.

Each reaction rate plotted in figure 10 is the average rate for the first half of each of the experiments (figures 7, 8 and 9). The rates are calculated in units of μmoles/l/sec based on the concentration of urea in the soil moisture. Soil moisture concentration was 24% for every experiment. Between 2°C and 45°C the Arrhenius plot is linear (figure 10) and the experimental activation energy for this interval is 9.8 kcal/mole. This compares quite well with an activation energy of 9.9 kcal/mole for bacterial urease below 25°C. The soil used to determine the activation energy had a pH of 6.4 compared to 6.7 for the experiment carried out by Larson and Kallio (1954) to determine the activation energy of bacterial urease. It is likely that a difference in pH of 0.3 units would not appreciably affect the activation energy of urease in soil. The most notable feature of the soil urease Arrhenius plot is the absence of either a discontinuity or a change in slope which suggests that soil urease probably does not change configuration appreciably as the temperature rises. Thus urease is probably stabilized by association with soil colloids.

The Urease Activity of Urea-Treated Soil

Soil urease activity can be stimulated by the addition of urea. Paulson and Kurtz (1969) added dextrose plus either ammonium or urea as a nitrogen source, and determined the effect on soil urease activity. The urea-treated soil had a higher urease activity but there was no difference between the two treatments in the total number of microorganisms or the number of ureolytic microorganisms. They

attributed the stimulation of activity to urea inducing the microorganisms to produce more urease. Roberge and Knowles (1966) added 400 lb. urea N/acre to the surface of a podzol. Two years later the activity was higher in the treated soil. Although the treated soil had a greater total number of microorganisms, the percentage of the total that was ureolytic was equal in both soils (Roberge and Knowles, 1967). The humus layer of a podzol has a high C/N ratio and would be quite deficient in nitrogen. Although the increase in activity for the treated soil can be attributed to an increase in the number of ureolytic microorganisms the effect is not selective since the general microbial population is also stimulated by the nitrogen provided.

When urea was applied to a Malmo clay loam no residual urea remained after an interval of two months and the activities in all the treatments were identical. Differences in activity were appreciable at 3½ months (figure 11). The urease activity was highest for the soil treated with 100 lb. urea N/acre, slightly lower for the untreated soil, and was lowest in the soil treated with 800 lb. urea N/acre. The pH was essentially the same in the untreated soil (6.20) and the soil treated with 100 lb. urea N/acre (6.25). The pH was found to be much lower (5.35) in the soil treated with the highest urea application which can be attributed to nitrification of the added nitrogen. The urease activity of soil decreases at both high and low pH values with the optimum activity occurring at a pH of approximately 7 (Vasilenko, 1962). The lower urease activity found in the soil receiving the highest urea treatment can be attributed to the lower pH of that soil.

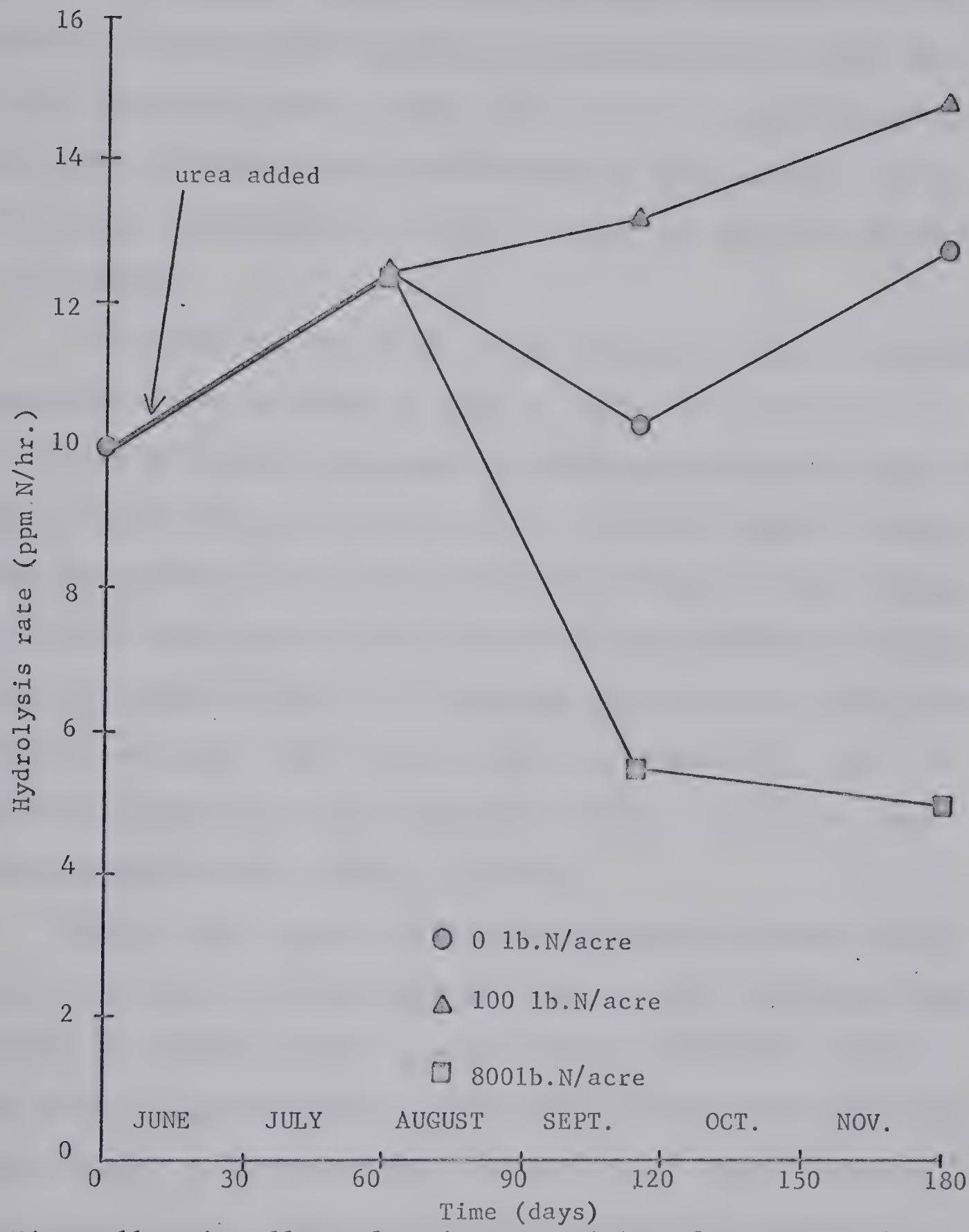


Figure 11. The effect of various quantities of urea on soil urease activity in a Malmo clay loam. Urease activity measured using the following conditions: 25°C, 24% moisture, and an initial substrate concentration of 200 ppm urea N. Soil samples 2-7 and 17-19 used; samples stored 1 week before activity measured.

The Urease Activity in Various Soils

The urease activity of soil is generally proportional to the number of ureolytic microorganisms (Roberge and Knowles, 1967) but is also affected by other factors. The activity can be related to the carbon or organic matter content which is just an indication of the general microbiological activity of which the ureolytic microflora is a portion.

The hydrolysis of urea in the LH horizon of the Brunisolic Gray Wooded was almost ten times as rapid as in the surface horizon of either the cultivated Gray Wooded or cultivated Chernozem (figure 12). The correlation between urease activity and percent organic carbon is poor when different soil types are compared (figure 12) but is good ($r = 0.99$) when horizons within one profile are compared (figure 15). Urease activity was found in all horizons of a Brunisolic Gray Wooded profile, and even as deep as the BC horizon (figure 14). The hydrolysis rate curves were approximately linear for all the lower horizons from the Ae to the BC (figure 13).

Other factors to be considered with respect to urease activity are soil pH and the cation exchange capacity. Soil pH has been shown to have an important effect on urease activity (Vasilenko, 1962). The effect of cation exchange capacity has not been investigated but may be useful as an indication of the ability of a soil to complex and retain urease. Additional work is necessary to correlate the urease activity with: (a) soil conditions, and (b) the number and type of ureolytic microorganisms.

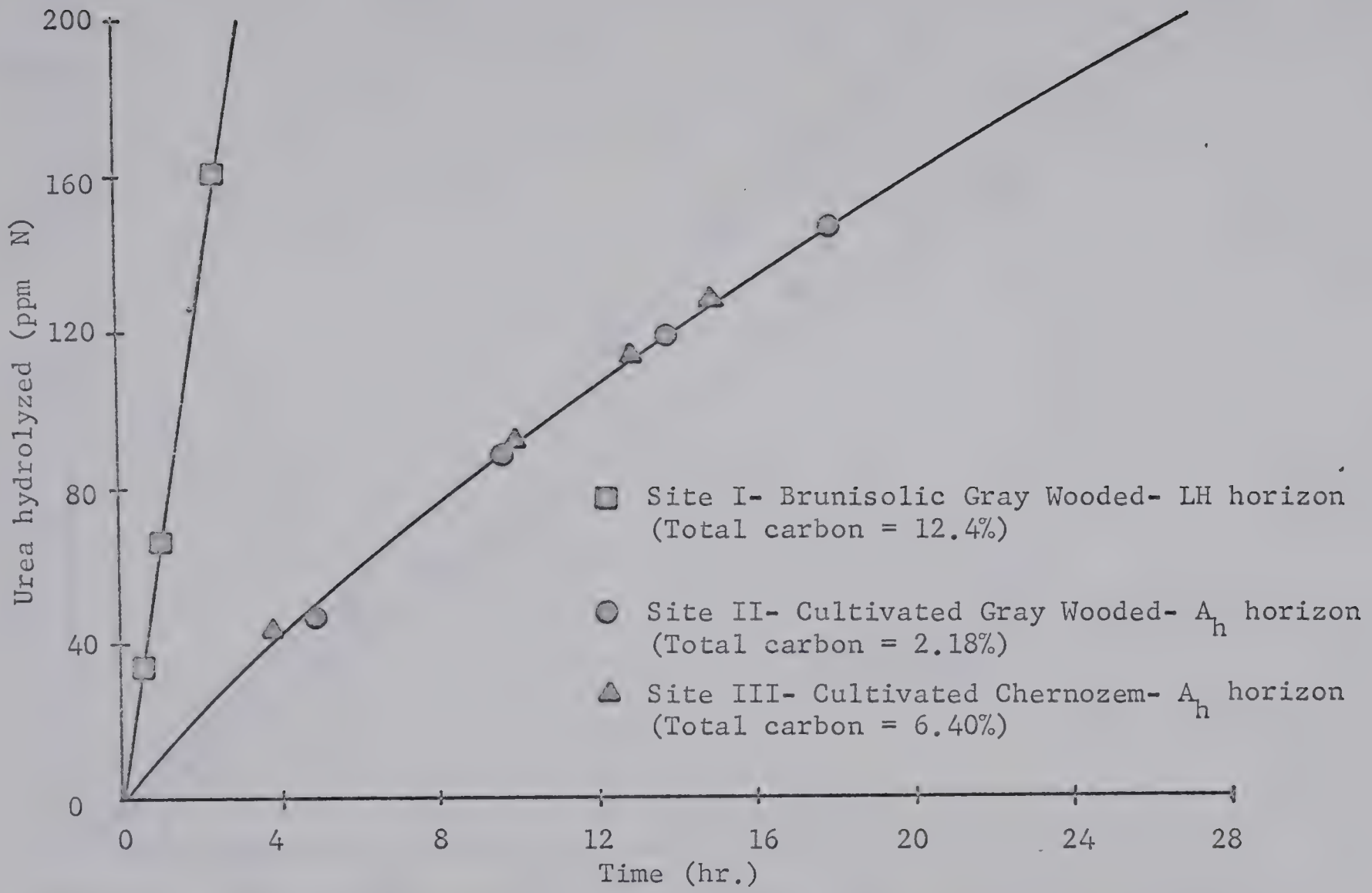


Figure 12. Urea hydrolysis in the surface horizons of several Alberta soils. Urease activity measured under the following conditions: 25°C, 50% moisture and an initial substrate concentration of 200 ppm urea N. Soil samples 7, 8, and 16 used.

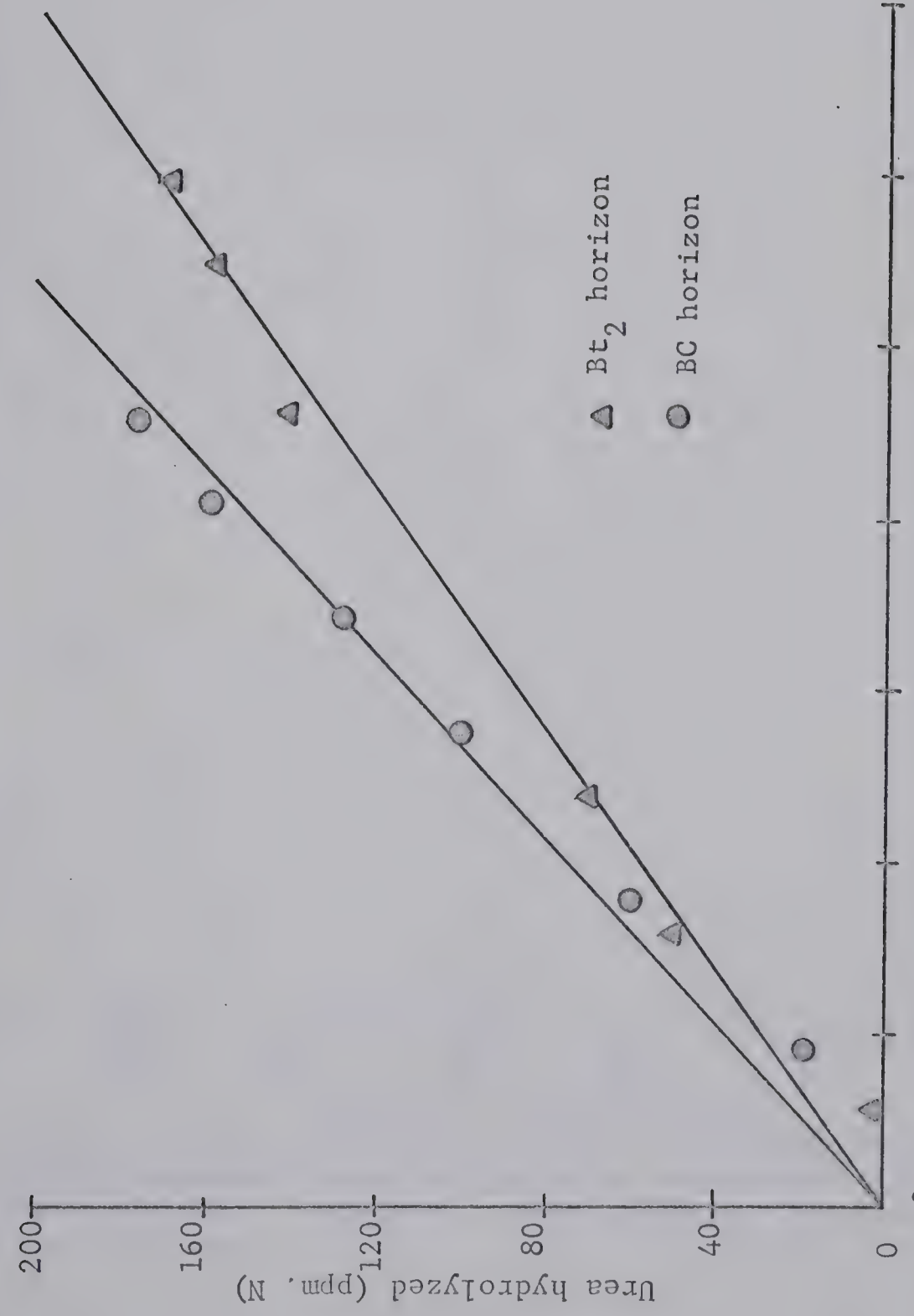


Figure 13. Hydrolysis of urea in the Bt_2 and BC horizons of Site I. Urease activity measured using the following conditions: 25°C, 50% moisture and an initial substrate concentration of 200 ppm urea N. Soil samples 14 and 15 used.

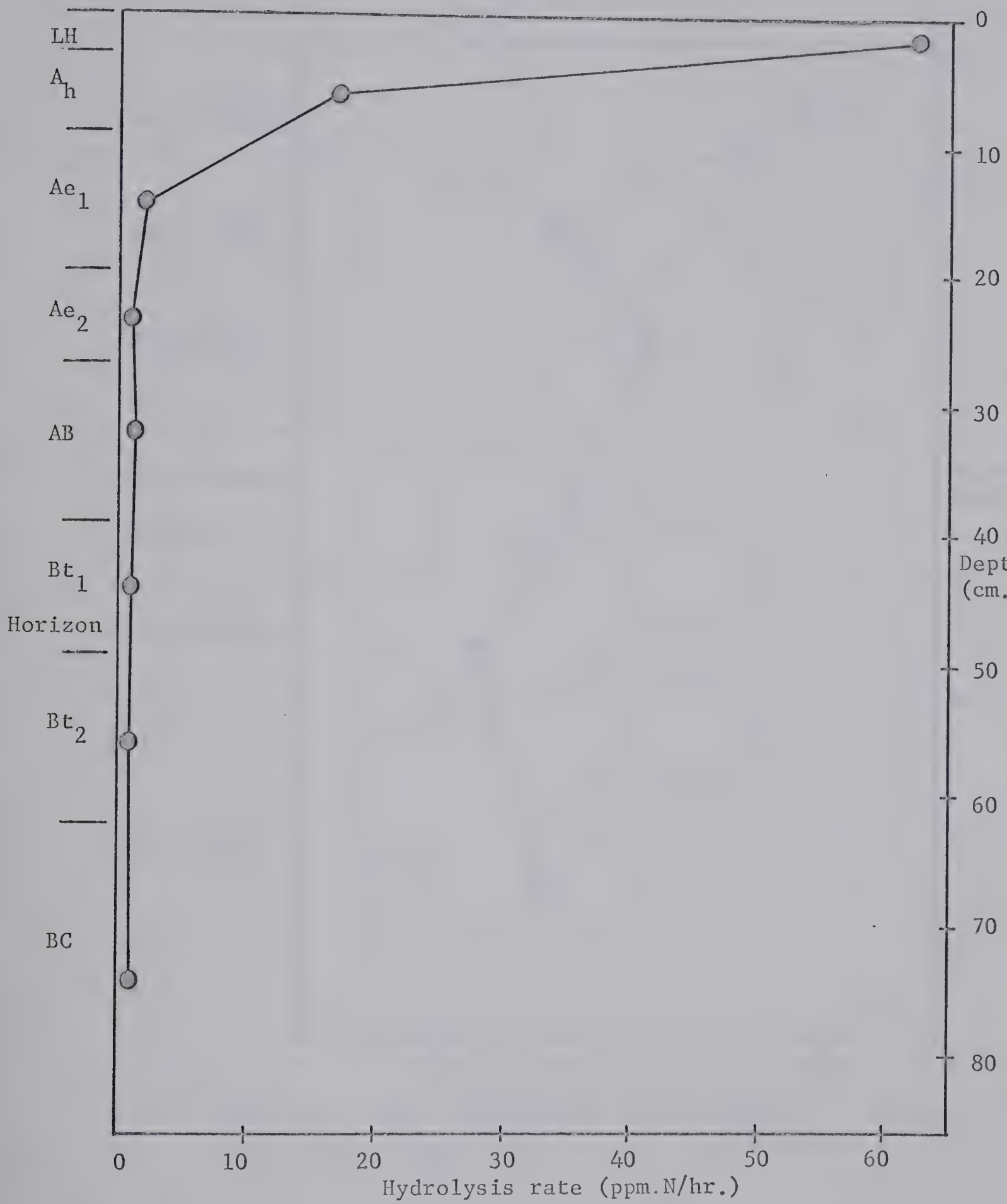


Figure 14. The variation of urease activity with depth at Site I. Urease activity measured using the following conditions: 25°C, 50% moisture, and an initial substrate concentration of 200 ppm urea N. Soil samples 8-15 used.

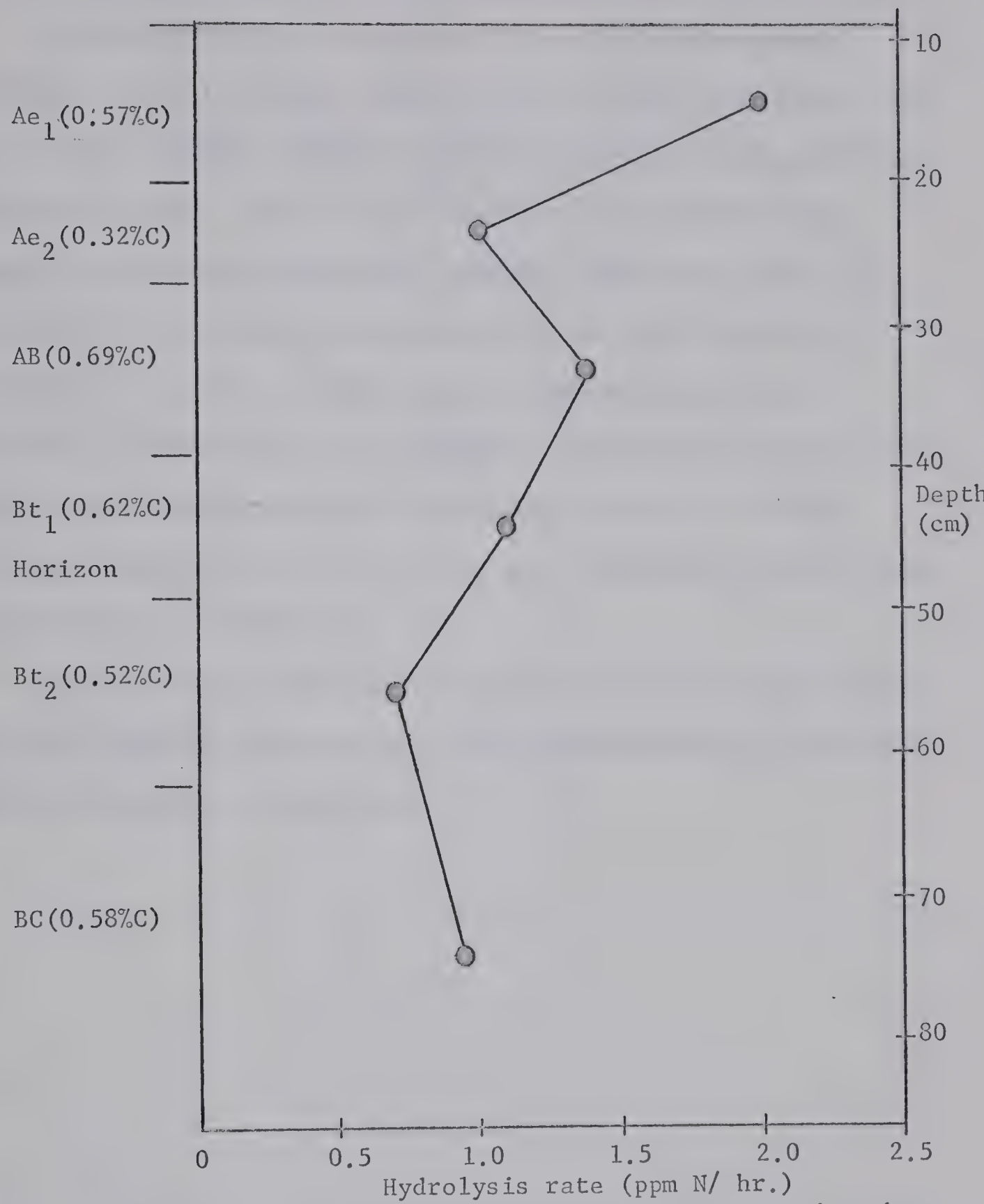


Figure 15. The variation of urease activity and carbon content with depth in the lower horizons of Site I.

Effect of Urease Inhibitors on the Hydrolysis Rate of Urea in Soil

Experiments were carried out with various known urease inhibitors as well as other compounds with related structures. In the untreated samples 100 ppm of urea N hydrolyzed during the 8 hour incubation period. Little or no inhibition was observed when compounds with an aldoxime group (-CH=NOH) were used (Table 2). Acetohydroxamic acid and hydroxyurea were the most effective inhibitors. Thus the $-\overset{O}{\overset{\|}{C}}-NHOH$ group is responsible for the inhibition exhibited by these compounds (Fishbein and Carbone, 1965). Sodium diethyldithiocarbamate is patented for use as a urease inhibitor with urea fertilizer (U. S. pat. #3,073,694) but was found to be relatively ineffective.

None of these inhibitors are agronomically practical due to the high inhibitor concentration (>100 ppm) required to achieve an appreciable degree of inhibition.

TABLE 2

The Inhibition of Urea Hydrolysis in Soil by Various Urease Inhibitors

Inhibitor	Inhibitor Concentration ppm	% Reduction in Hydrolysis
Acetohydroxamic acid	100	23
Acetohydroxamic acid	25	12
Acetohydroxamic acid	5	3
Hydroxyurea	100	17
Sodium diethyldithiocarbamate	100	8
3-pyridinealdoxime	100	6
2-pyridinealdoxime	100	0
Furfuraldoxime	100	0

V. SUMMARY AND CONCLUSIONS

The effect of certain variables on the hydrolysis rate of urea in soil has been studied. The following conclusions have been drawn.

1. A method for the extraction of urea from soil for analysis has been improved.
2. The effect of urea concentration on the hydrolysis rate of urea in soil was not fully defined but the rate was found to be linearly dependent on concentration.
3. The urease activity of stored soil increased initially and then decreased slowly over a long period of time.
4. Moisture concentrations above field capacity had little effect on the hydrolysis rate of urea in soil.
5. The Arrhenius plot for the hydrolysis of urea in soil was found to be linear from 2°C to 45°C. The experimental activation energy for this reaction is 9.8 kcal/mole.
6. The urease activity of soil was stimulated by a moderate application of urea but was depressed by a heavy application. The reduction in activity can be attributed to the low pH resulting from nitrification of the added nitrogen.
7. Urease activity studied in all horizons of a Brunisolic Gray Wooded soil profile showed a good correlation ($r = 0.99$) with the organic carbon content.
8. Acetohydroxamic acid was found to be the most effective inhibitor of soil urease. However none of the compounds tested are effective enough to be agronomically practical.

More work is necessary to clarify the effect of urea concentration on the hydrolysis rate, and additional compounds should be tested for urease inhibition in soil. An ecological study should be carried out to determine the relationships between urease activity, soil conditions and the ureolytic microflora.

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APPENDIX I

PROFILE DESCRIPTION OF SITE I

LOCATION: N W 7 - 51 - 21 W 4

VEGETATION: Aspen Poplar (Populus tremuloides)

CLASSIFICATION: Brunisolic Gray Wooded

Horizon	Depth, cm.	Color (Moist)	Texture	Structure	Consistence (Moist)
LH	3	10 YR 2/1			
Ah	6	10 YR 2/1	L	Granular	Soft, friable
Ae_1	11	10 YR 5/4	SL	Medium platy	Soft, friable
Ae_2	7	10 YR 6/3	SL	Medium platy	Soft, friable
AB	12	10 YR 5/4	CL	Medium subangular blocky	Firm
Bt_1	10	10 YR 4/3	C	Coarse prismatic subangular blocky	Very firm
Bt_2	13	10 YR 4/3	C	Coarse prismatic subangular blocky	Very firm
BC	24	10 YR 3/3	C	Coarse subangular blocky	Very firm

APPENDIX II

CHEMICAL AND PHYSICAL ANALYSES FOR SITES I, II AND III

Site	Horizon	pH	Total CEC me/100 g	Total Carbon %	Mechanical Analyses Sand %	Silt %	Clay %
I	LH	6.25	47	12.4	-	-	-
I	Ah	6.20	24	4.75	47	39	14
I	Ae	6.20	6	0.57	55	33	12
I	Ae	5.70	8	0.32	52	29	19
I	AB	5.40	21	0.69	38	22	40
I	Bt	5.10	21	0.62	39	22	39
I	Bt	5.00	21	0.52	39	23	38
I	BC	5.00	20	0.58	39	24	37
II	Ah	6.40	15	2.18	49	33	18
III	Ah	6.40	37	6.40	29	39	32

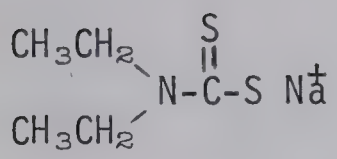
APPENDIX III

DATE OF SAMPLING AND TREATMENTS FOR SOIL SAMPLES FROM SITES I, II AND III

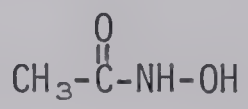
Sample No.	Site	Horizon	Date Taken	Other Treatments
1	III	Ah	May 23, 1969	
2	III	Ah	July 23, 1969	800 lb. urea N/acre
3	III	Ah	July 23, 1969	100 lb. urea N/acre
4	III	Ah	July 23, 1969	
5	III	Ah	Sept. 15, 1969	800 lb. urea N/acre
6	III	Ah	Sept. 15, 1969	100 lb. urea N/acre
7	III	Ah	Sept. 15, 1969	
8	I	LH	Sept. 18, 1969	
9	I	Ah	Sept. 18, 1969	
10	I	Ae_1	Sept. 18, 1969	
11	I	Ae_2	Sept. 18, 1969	
12	I	AB	Sept. 18, 1969	
13	I	Bt_1	Sept. 18, 1969	
14	I	Bt_2	Sept. 18, 1969	
15	I	BC	Sept. 18, 1969	
16	II	Ah	Sept. 18, 1969	
17	III	Ah	Nov. 18, 1969	800 lb. urea N/acre
18	III	Ah	Nov. 18, 1969	100 lb. urea N/acre
19	III	Ah	Nov. 18, 1969	

APPENDIX IV

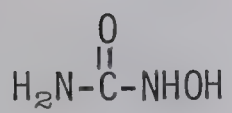
UREASE INHIBITORS EMPLOYED IN STUDY



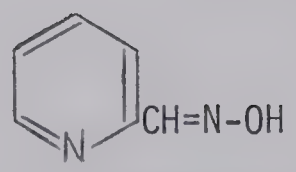
Sodium diethyldithiocarbamate



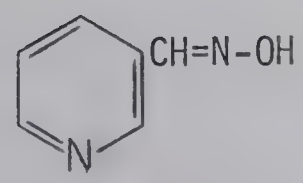
Acetohydroxamic acid



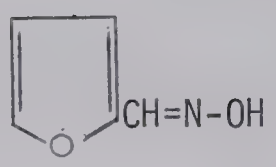
Hydroxyurea



2-pyridinealdoxime



3-pyridinealdoxime



Furfuraldoxime

APPENDIX V

STANDARD CURVE FOR UREA